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The effect of amorphous pyrophosphate on calcium phosphate cement resorption and bone generation☆

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ABSTRACT

Pyrophosphate ions are both inhibitors of HA formation and substrates for phosphatase enzymes. Unlike polyphosphates their hydrolysis results simultaneously in the complete loss of mineral formation inhibition and a localised elevation in orthophosphate ion concentration. Despite recent advances in our knowledge of the role of the pyrophosphate ion, very little is known about the effects of pyrophosphate on bone formation and even less is known about its local delivery. In this work we first developed a self setting pyrophosphate based calcium cement system with appropriate handling properties and then compared its in vivo degradation properties with those of a non-pyrophosphate containing control. Contrary to expectation, the presence of the pyrophosphate phase in the cement matrix did not inhibit mineralisation of the healing bone around the implant, but actually appeared to stimulate it. *In vitro* evidence suggested that enzymatic action accelerated dissolution of the inorganic pyrophosphate ions, causing a simultaneous loss of their mineralisation inhibition and a localised rise in supersaturation with respect to HA. This is thought to be a rare example of a biologically responsive inorganic material and these materials seem to be worthy of further investigation. Bioceramics to date have mainly been limited to orthophosphate, silicate and carbonate salts of calcium, here we report the successful application of a pyrophosphate material as a degradable osteoconductive bone repair cement.

1. Introduction

Calcium orthophosphate based bone graft replacements have been investigated as potential bone graft replacements since 1920 when tricalcium phosphate was injected into an animal model [1]. During the 70's there was a concerted effort to develop new calcium phosphate grafts based on tricalcium phosphate or hydroxyapatite (HA) monoliths [2,3]. Despite 40 years of further research, HA has not replaced autologous tissue as the surgical 'gold-standard'. The clinical success of HA can be attributed to the fact that it forms a bond with both hard and soft tissues and is osteoconductive [4]. One of the drawbacks of using HA is that, in its dense form, it remains in the body for a prolonged period following implantation [5] and its brittle nature means that it presents a long-standing risk of catastrophic failure [6]. Some workers have attempted to solve this problem by incorporating macroporosity (>200 μm) to allow bone in-growth [7]. While the original rationale behind using HA as bone replacements was clear, the principle itself is somewhat crude. The HA crystallites in bone are typically nanoscopic (100 nm in length, thickness 4–6 nm, and breadth 30–45 nm [8]) and incorporate numerous substitutions, whereas sintered HA and β-TCP (β-tricalcium phosphate; $Ca_3(PO_4)_2$) are microcrystalline and consequently exhibit a correspondingly low specific surface area. Furthermore, although bone is formed of 60–70 vol% HA, the collagen matrix and other proteins in bone play important biological and structural roles. Therefore, other than a compositional similarity, HA bone graft substitute typically has little mechanical or biological similarity to bone. Consequently, a number of other groups have attempted to

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develop new grafting materials using other osteoconductive calcium salts more soluble in physiological conditions such as brushite [9–12]. Although some success has been reported, brushite grafts tend to hydrolyse to form HA [13], which can also result in long-term implant stability and therefore present a prolonged risk of implant failure. There are several reports in the literature of brushite-based calcium phosphate cements being chemically modified to prevent the hydrolysis reaction from occurring. Newberryite [14], for example, has been added to brushite cement and has been shown to prevent hydrolysis from occurring *in vitro*. Unfortunately, these results could not be reproduced *in vivo* [15]. We have also modified brushite-based cements using pyrophosphoric acid as a reactant [12,16,17]. This modification enabled the production of a cement containing an amorphous pyrophosphate phase, which was shown to prevent the hydrolysis reaction for over 90 days *in vitro* [16]. Pyro or diphosphate ($P_2O_7^{4-}$) ions are inhibitors of HA crystal formation and are essential in the control of biomineralisation [17–19], their potent mineralisation inhibiting activity has been exploited to soften water. Importantly, although pyrophosphates inhibit mineral formation [18], active osteoblasts secrete alkaline phosphatase (ALP), which can hydrolyse pyrophosphate ions (Equation (1)), which simultaneously causes loss of this ion's potent inhibiting effect on HA formation and supersaturates the extra-cellular fluid with orthophosphates that induce mineralisation [19]. This mechanism has been shown to be critical to the control of bone mineralisation. With the exception of the highly insoluble and thermally stable phase β-dicalcium pyrophosphate [20], the pyrophosphates are relatively unexplored in bone graft replacement applications, despite decades of research demonstrating the role of this ion in bone.

$$P_2O_7^{4-} + H_2O \rightarrow 2PO_4^{3-} + 2H^+ \quad (1)$$

Here we have investigated whether the amorphous pyrophosphate phase can prevent brushite hydrolysis and consequently long-term cement stability *in vivo*. Following periods of implantation, cement samples were retrieved and characterised with respect to composition using ion chromatography and X-ray diffraction. Sections were made of the implant site and the extent of implant resorption was determined using histomorphometry. Finally, an *in vitro* model was used to systematically evaluate how the enzyme alkaline phosphatase interacts with amorphous calcium pyrophosphate phase.

2. Materials and methods

2.1. Cement formulation

A systematic study was undertaken to determine the liquid compositions at which setting pyrophosphate cements could be produced while maximising amorphous calcium pyrophosphate content. All cement pastes were formed from the mixture of β-TCP (Plasma-Biotal, Derbyshire, UK), pyrophosphoric acid, orthophosphoric acid (Sigma–Aldrich, Gillingham, UK) and double distilled water at a constant powder to liquid ratio (P:L) of 1.50 g/mL. Cement pastes were formed where the water constituted of between 0 and 90 wt% of the liquid phase (at 5 wt% increments). At each water content, the proportions of pyrophosphoric and orthophosphoric acids were varied so that every possible liquid composition (at 5 wt% increments) was produced. To find the final setting times exhibited by the cements a needle (1.05 mm diameter and 454 g mass) was applied to the surface of the cement. The point at which this needle no longer indented the surface was the final setting time. All measurements were made in ambient conditions and were repeated three times for each cement formulation investigated. From the resultant data the compositions were grouped into those that when mixed with β-TCP (powder to liquid ratio 1.50 g/mL) set in a.) less than 30 s, b.) 30 s to 30 min, c.) 30 min to less than 24 h and d.) non-setting formulations. A ternary plot was then constructed to show the liquid compositions resulting in the formation of cements setting fully in these ranges. Once the ternary plot was completed the entire experiment was repeated to ensure accuracy.

2.2. X-ray diffraction and nuclear magnetic resonance

The compositions of the hardened cements were determined using X-ray diffraction (XRD) and nuclear magnetic resonance (NMR). Dried cement samples were powdered using a pestle and mortar. For XRD, the powder was placed between two pieces of magic™ tape (3M, Minnesota, USA) and attached to the sample holder, which was mounted in the X-ray diffractometer (Siemens D5000, Munich, Germany). X-ray powder diffraction data were collected using Cu $K\alpha_1$ radiation from a powder X-ray diffractometer, fitted with a Ge primary beam monochromator and aligned in transmission mode. For phase analysis each data set was collected from 5° to 100° 2θ with step size 0.02°, each step requiring 9 s, such that patterns required approximately 12 h to collect. A multiphase, whole pattern Rietveld analysis using the General Structure Analysis Suite (GSAS) of programs was employed to determine the crystalline phase compositions of the samples. Structural models for $CaHPO_4 \cdot 2H_2O$, $CaHPO_4$, β-$Ca_3(PO_4)_2$, β-$Ca_2(P_2O_7)$, $Ca_2P_2O_7 \cdot 2H_2O$, and $Ca_{10}(PO_4)_6OH_2$ [21–26] were obtained from the literature references and a linear interpolated background function was utilised, together with a pseudovoigt peak shape function. The pyrophosphate contents of cement formulations one to eleven were determined by quantitative NMR of powdered cement. High-resolution solid state ^{31}P NMR experiments were performed at 121.51 MHz using an NMR spectrometer (CMX Infinity 300 spectrometer, Chemagnetics, CO, USA) with a Chemagnetics 4 mm triple-resonance magic angle spinning (MAS) probe. All spectra were recorded using direct polarisation with ^1H decoupling (with a decoupling field strength of 100 kHz), magic angle spinning (with a spinning frequency of 7000 ± 2 Hz) and a set sample temperature of 25 °C. The spectra were referenced relative to 85 wt% orthophosphoric acid at 0 ppm. To ensure that the ^{31}P MAS NMR spectra of the cement samples were quantitative ^{31}P spin-lattice relaxation times (T_1) were first determined for samples of the components of the cements using the saturation-recovery technique. The high-resolution solid state ^{31}P NMR spectra of the cement samples were then recorded using a recycle delay of greater than five times the longest value T_1 determined for the individual cement components. The composition of the cements could then be determined by fitting the spectra using the spectrometer software to obtain reliable peak intensities.

2.3. Determination of orthophosphate:pyrophosphate

In order to determine the proportions of the hardened cement consisting of orthophosphate and pyrophosphate phases, approximately 25 mg of cement were dissolved in 100 mL nitric acid (100 mM). The resulting solution was diluted ten times with double distilled water before analysis using an ion chromatography system (ICS-2500, Dionex, Sunnyvale, CA, USA) equipped with an appropriate analytical column (IonPac AS16, Dionex, Sunnyvale, CA, USA).

2.4. In vivo experimentation

To evaluate biological response to the material and cement resorption, samples were implanted in an ovine model. The pyrophosphate modified cement used in the study was formed by combining β-TCP with pyrophosphoric acid (540 mg) and double distilled water (720 mg) at a powder to liquid ratio of 2.25 g/mL. The orthophosphate brushite cement was formed by the combination of β-TCP with orthophosphoric acid (2 M) containing trisodium citrate (50 mM) at a powder to liquid ratio of 1.75 g/mL. The cements were cast into PTFE split moulds to form hardened cement cylinders (diameter 6.4 mm and length 12 mm). The samples were stored at 37 °C and 100% relative humidity for 24 h prior to sterilisation by gamma irradiation. The cement cylinders (four replicates per time-point) were implanted into the tibiae of eighteen female Blue Faced Leicester cross Suffolk sheep of approximately four years in age according to a randomisation schedule. Briefly, anaesthesia was induced with thiopentone sodium (5%) which was administered 'to effect' by intravenous injection. An incision was made in the medial side of the proximal tibia. A defect (6.4 mm diameter, 12 mm deep) was then created with an orthopaedic drill, with at least one intermediate-sized drill used to reduce the chance of thermal necrosis to the bone. Bone debris was removed by saline irrigation and the defect flushed with local anaesthetic. The defects were filled with test material according to the randomisation schedule. To enable the location of the defect site two K-wires were inserted either side of it. Each K-wire was implanted at a distance of approximately 8 mm from the centre of the implant so that the wires and the centre of the implant were aligned in a straight-line. The K-wires were inserted at a depth not greater than 30 mm. This procedure was then repeated in the bilateral leg. The muscle, fascia, subcutaneous tissue and skin were closed in a standard surgical manner using resorbable sutures. Calcein was administered 24–48 h (10 mg/kg) after surgery. Alizarin C-one marker was administered at one, two and four months following implantation in the sheep (30 mg/kg) for the three, six and twelve month time-points, respectively. Oxytetracycline was given (30 mg/kg) one week prior to sacrifice. All animals were euthanised using Pentoject. Following gross pathology observations the implant site/tibial plateau was removed and the surrounding bone trimmed down. Some surrounding undamaged bone was retained so that histological analysis of the undamaged bone could be performed. All samples were fixed in alcohol (70%) at 4 °C. The implants were then bisected into two halves using a low-speed diamond saw. The cortical half of the explant was embedded into a methyl methacrylate resin. The resin blocks were sectioned using the Leica diamond microtome saw. Sections were cut (200 μm thickness) and adhered to plastic slides. The sections were ground down to approximately 100 μm. Two resin sections were prepared, one for evaluation by fluorescent microscopy, the other was stained with

alizarin red and used to evaluate bone growth. Image capture was performed using the using the Leica FLZ III Stereomicroscope coupled to a JVC digital camera (KY-F1030). Images of normal trabecular bone (stained with alizarin red) were also captured. These were used to calculate the area of bone that would be observed in a defect that had undergone complete repair. Image analysis of the alizarin red stained sections was carried out using Image-Pro Plus version 5.0 software. Calibration was performed using a 10 mm graticule as follows. Measurements were performed using a colour segmentation technique presuming the diameter of the original implant to be 6.4 mm. Newly formed bone was coloured green. Following colour segmentation, the proportion of new bone was determined using Excel (Microsoft, USA).

2.5. *In vitro evaluation of amorphous calcium pyrophosphate dissolution*

Synthesis of amorphous $Ca_2P_2O_7{\cdot}4H_2O$ was achieved using a precipitation reaction involving the addition of 200 mL of a 0.2 mol L^{-1} solution of potassium pyrophosphate ($K_4P_2O_7{\cdot}3H_2O$, Sigma Aldrich, Gillingham, UK, 97% purity) to 200 mL of 1.0 mol L^{-1} calcium chloride ($CaCl_2{\cdot}2H_2O$, Sigma Aldrich, Gillingham, UK, 97% purity) aqueous solution under vigorous stirring at room temperature [27].

In vitro experimentation was undertaken to evaluate the interaction between amorphous calcium pyrophosphate and ALP enzyme. 10 mg of amorphous calcium pyrophosphate was incubated with 20 U ALP (Sigma Aldrich, Gillingham, UK) in 10 mL Tris HCL (Sigma–Aldrich, Gillingham, UK) solution. The solutions were placed in a water bath at 37 °C under vigorous stirring. The concentration of PO_4^{3-} ion released into solution was determined by ICP. The samples were taken every 15 min in the first hour and every hour for the remaining 6 h. 1 mL of the solution was removed before the medium was centrifuged at 1000 r/min for 2 min, then 1 mL of Tris HCL solution with the same concentration of ALP was then added to the medium. For ICP detection, all the samples were diluted to 5 mL using distilled water before testing. For comparison, the experiment was done in the Tris HCL solution without ALP under the same condition.

3. Results

3.1. *Cement formulation parameters*

A systematic study was undertaken to identify liquid compositions that facilitated the production of cements that exhibited clinically relevant setting times of between 30 s and 30 min. The resulting data was aggregated to form a ternary liquid composition diagram (Fig. 1a). There were two distinct regions in which the liquid compositions allowed setting in 30 s–30 min. The material produced using >60 wt% pyrophosphoric acid dispersed on immersion in water. The set cements contained varying proportions of brushite, monocalcium phosphate monohydrate (MCPM), monetite, unreacted β-TCP and a hydrated calcium pyrophosphate. The presence of the calcium pyrophosphate was detected by nuclear magnetic resonance (NMR) (Fig. 1b) and ion chromatography, but was not detected by X-ray diffraction (XRD), suggesting that it exhibited an amorphous structure. Depending on liquid composition either purely orthophosphate (1–3) or pyrophosphate (4–11) containing matrices were formed (Fig. 1a,b (1–3) and (4–11), respectively). Formulations 1–3 were shown to consist entirely of MCPM and so were excluded from further study. By further optimising the cement formulation, it was possible to produce a material that consisted of as much as 28 wt% amorphous calcium pyrophosphate (Fig. 1b) and yet exhibited a compressive strength of 25 ± 2 MPa, in the same magnitude as cancellous bone and other inorganic bone cements [28].

3.2. *In vivo experimentation*

The cement formulation which consisted of the highest proportion of amorphous calcium pyrophosphate (28 wt%) was implanted into the proximal tibia in the hind legs of sheep for periods of three, six and twelve months after which the degradation of the pyrophosphate cement and bone formation were compared with an orthophosphate cement and a trabecular bone control. The orthophosphate control cement was initially resorbed and replaced by new bone at a similar rate to the pyrophosphate cement (Fig. 2a and b) (6 and 5 area% new bone respectively) with

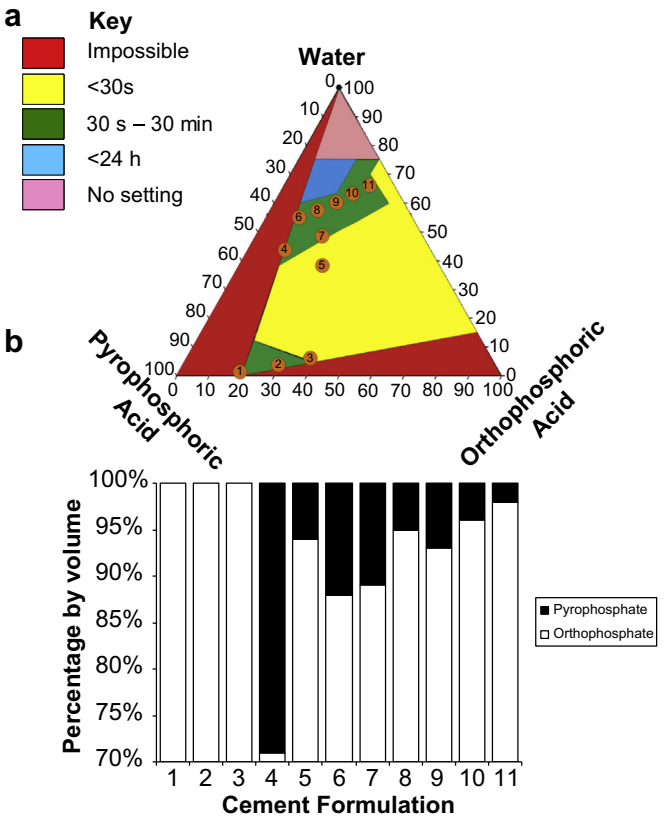

Fig. 1. a. A ternary liquid-composition setting diagram. The cements were grouped according to their setting times and eleven cement formulations (marked on the diagram) were selected for compositional characterisation using NMR. b. The proportions of cement formulations 1–11 comprised by pyrophosphate and orthophosphate components. In the case of the cement formulations consisting of <55 wt% pyrophosphoric acid (marked 4–11) an increase in the concentration of pyrophosphoric acid resulted in an increase in the proportion of amorphous calcium pyrophosphate precipitated. However, in the case of the cements formed liquid components containing pyrophosphoric acid concentrations higher than 78 wt% (marked 1–3) there was no calcium pyrophosphate formed, rather the cement consisted of MCPM, which was probably precipitated as a result of the low pH and calcium to phosphorus ratio of the cement mix.

the formation of a layer of new bone across the surface of each material. Following six and twelve months of implantation, however, little further reduction in the area of the orthophosphate cement was noted and consequently the proportion of new bone formed in the defect was limited (14 area% after 12 months). The cement remained as a dense core in the centre of the bone defect. This was in contrast to the pyrophosphate cement, which degraded at a similar rate over the first three months of the study (replaced by 5 area% new bone), but over the remainder of the study continued to degrade from it's periphery and was replaced with newly formed bone. Following 12 months the pyrophosphate cement had degraded more extensively than the orthophosphate cement, with the formation of 33 area% new bone, which was comparable with natural trabecular bone (24–36 area%, Fig. 2b). The surface of the cement core was crenellated with evidence of new bone formation in regions of ceramic degradation.

3.3. *Compositional change during degradation studies*

In order to determine the origin of the difference in degradation behaviour, the compositions of the retrieved cement implants were characterised using a combination of XRD and ion chromatography. XRD data (Fig. 3) showed that the orthophosphate cement contained minimal brushite in the defect after three months, with the

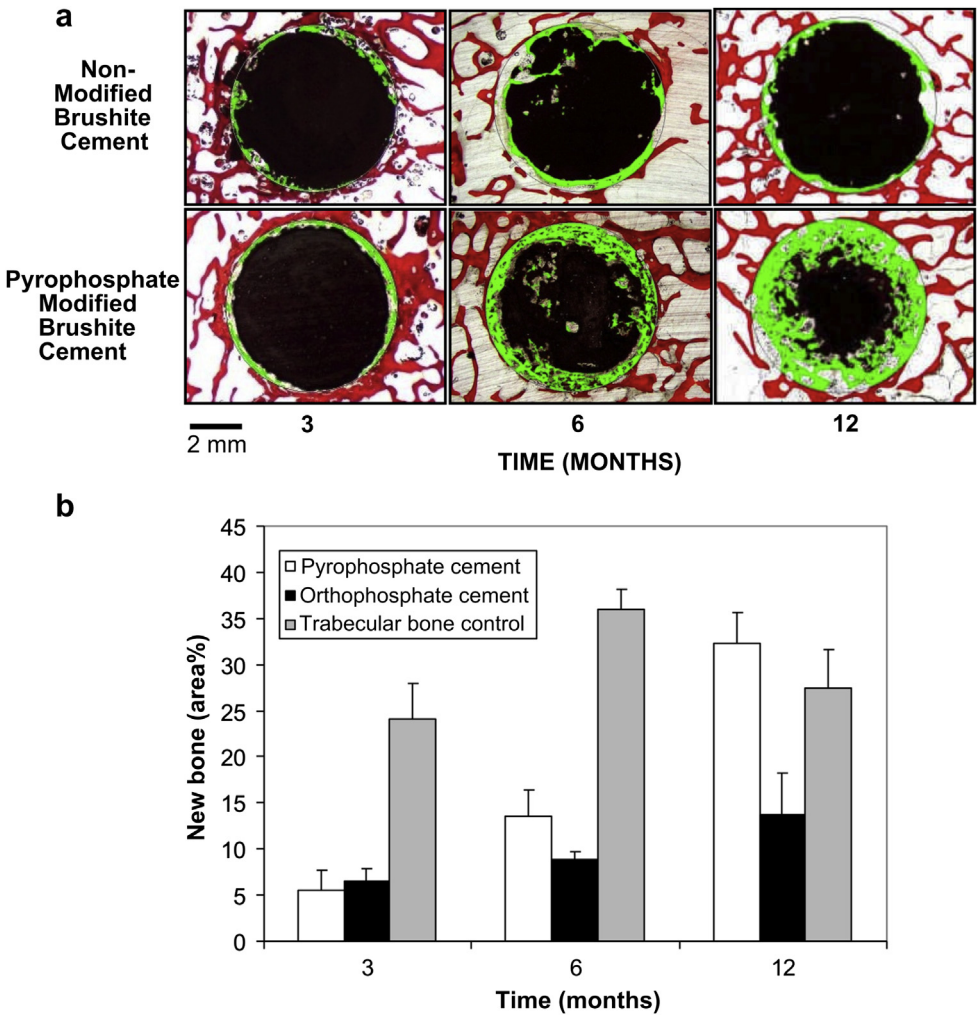

Fig. 2. a. Histological sections through the implanted cement and surrounding bone. The alizarin red stained sections through the orthophosphate and pyrophosphate modified brushite cement following implantation in the tibia of a sheep for three, six and twelve months. In all cases the original cancellous bone is stained in red, new bone in green pseudo-colouring and the cement is unstained. b. The percentage area of new bone in the cement cavity following implantation for 3, 6 and 12 months. The error bars shown represent the standard error in each condition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

only apparent crystalline phase being β-TCP. In contrast, the pyrophosphate modified cement contained significant amounts of brushite even after twelve months. The calcium pyrophosphate content of the ceramic was determined after each time-point and this work demonstrated that the weight fraction of the material that constituted calcium pyrophosphate diminished until after 12 months the proportion of material contributed by calcium pyrophosphate reduced to 30 wt% of the starting value (Fig. 4). This reduction in the proportion of cement contributed by the calcium pyrophosphate phase demonstrates that it was preferentially dissolved from the implanted material. To determine whether this preferential dissolution of calcium pyrophosphate could be attributed to enzyme mediated pyrophosphate hydrolysis, an *in vitro* experiment was undertaken where an amorphous calcium pyrophosphate (as synthesised in accordance with the method of Slater et al. [27]) was exposed to ALP. The resulting data clearly demonstrated an increase in the concentration of PO_4^{3-} ions in the presence of ALP (Fig. 5a) thus confirming the pyrophosphate released into solution was cleaved to form orthophosphate ions as compared with the control buffer solution. Interestingly, these high levels of phosphate and calcium ions should provide for local supersaturation with respect to hydroxyapatite precipitation, however our animal studies indicate that any such precipitation appears to have occurred within the natural process of new bone deposition and not as isolated HA deposits. To identify whether or not this process was surface mediated, an experiment was undertaken whereby calcium pyrophosphate dihydrate crystals were aged within a visking tubing that prevented the access of ALP to the surface of the calcium pyrophosphate crystals (Fig. 5b). Even when ALP was isolated from the surface of the crystal, there was a notable acceleration in dissolution when compared with the control, suggesting that this process was not surface mediated.

4. Discussion

Sparingly soluble calcium phosphate salts have been widely investigated for application as resorbable bone replacement materials. β-TCP forms the basis of a number of commercially available bone graft replacements [2], but as it does not form in ambient conditions, it is not widely used for reconstruction following trauma. Calcium phosphate cements can harden to form a matrix that consists, in the most part, of brushite [10,11]. As this material is several orders of magnitude more soluble than hydroxyapatite, it can be resorbed by a combination of cell-mediated degradation and simple dissolution [15]. The metastable nature of the salt, however, means that it has been shown to hydrolyse to form a poorly

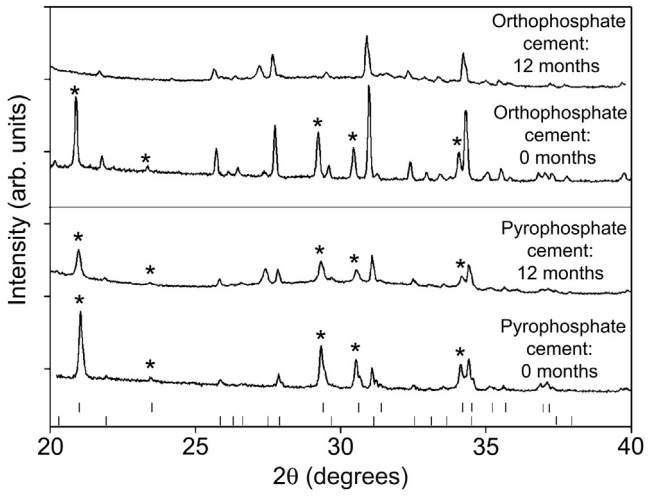

Fig. 3. X-ray diffraction patterns of the orthophosphate and pyrophosphate brushite cements following implantation for 0 and 12 months. Peaks that are marked with an * indicate the presence of brushite in the cement matrix. Reflection markers for brushite (upper) and β-TCP (lower) are also included. The pyrophosphate modified cement contained brushite following 12 months of ageing, whereas the orthophosphate brushite cement did not.

crystalline hydroxyapatite which slows or prevents resorption *in vitro* and *in vivo* [13,15,16]. Efforts have been made to inhibit this hydrolysis reaction and prevent long-term stability of the grafts, for example, by adding a magnesium salt (Newberryite) into the hardened matrix [14]. These approaches, however, have had no success in preventing long-term stability *in vivo*. We have previously reported the formulation of a material using combination of a sparingly soluble calcium phosphate (α-TCP, β-TCP or TTCP) and a condensed phosphoric acid [12,15] to form an amorphous calcium pyrophosphate in the cement matrix. This material was shown to be resistant to hydrolysis over a period of 90 days of ageing using an *in vitro* model [16]. Here we have demonstrated that this material is not only resistant to hydrolysis, but also seems to stimulate bone generation around the implant.

Over the course of the study, the amorphous calcium pyrophosphate in the pyrophosphate cement effectively prevented brushite hydrolysis. As the pyrophosphate cement was more extensively resorbed than the orthophosphate cement control, we may speculate that the significant hydrolysis of brushite to less

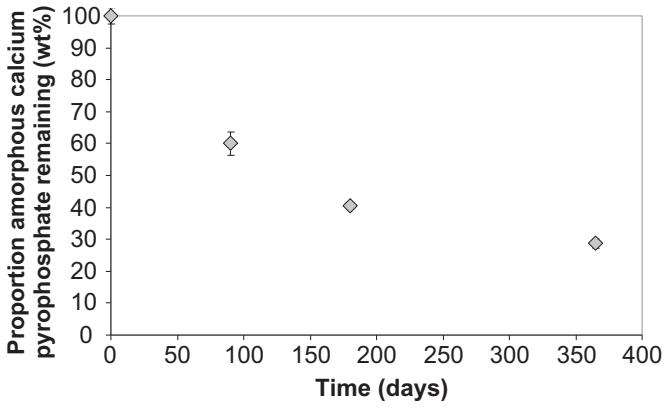

Fig. 4. The quantity of calcium pyrophosphate in the pyrophosphate modified brushite cement after 0, 3, 6 and 12 months of implantation. Although there was a steady reduction in the quantity of amorphous calcium pyrophosphate present in the cement with time, there was no deleterious effect on the deposition of new bone. *In vitro* experiments confirmed that as the pyrophosphate ions were released into solution, they were broken down by ALP, an enzyme with a known pyrophosphatase function.

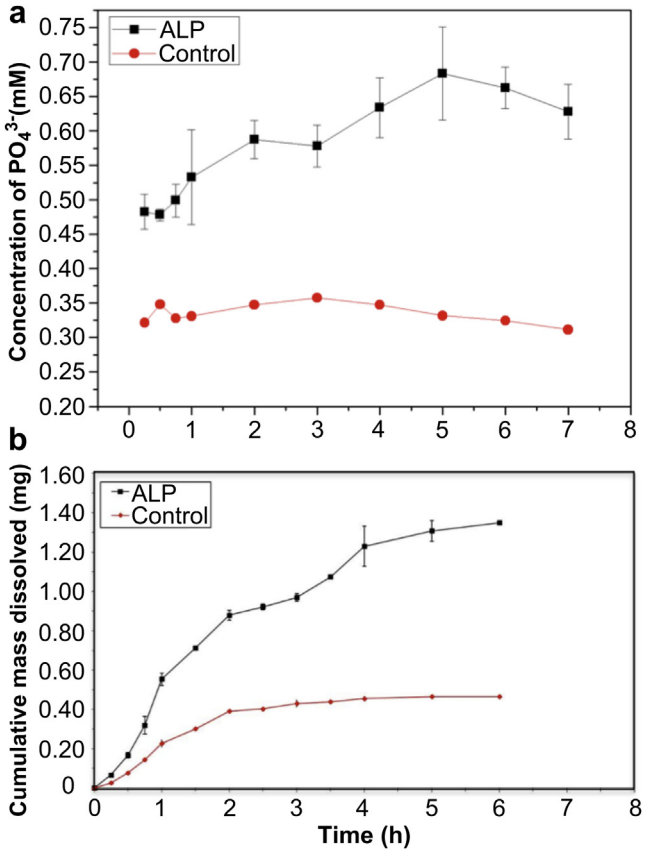

Fig. 5. The action of ALP on pyrophosphate ions accelerating calcium pyrophosphate dissolution. a. Orthophosphate concentration in solution over a period of 6 h immersion. Amorphous calcium pyrophosphate was immersed in ALP and non-ALP containing solutions (Control), and dissolution was monitored. In the presence of ALP, the phosphate concentration was substantially higher than in its absence indicating that ALP acted to accelerate dissolution. b. Calcium pyrophosphate crystal dissolution when ALP has or does not have access to the crystal surface. There was no difference in crystal dissolution when ALP was separated from the cement surface by a visking tubing (Control) and had access to the surface (ALP) suggesting that the accelerated dissolution process was not surface mediated.

resorbable material, such as poorly crystalline HA, may explain the reduced resorption of the orthophosphate cement. Pyrophosphate ions have long been identified as potent inhibitors of apatite formation *in vivo* [18], a finding that led to the use of hydrolysis resistant phosphonate analogues as effective osteoporosis treatments [29], and even today a confusion exists between inhibiting apatite formation *in vitro* and inhibiting bone formation *in vivo*, since in addition to poisoning apatite nucleation, they have also been shown to cause the apoptosis of osteoclast (bone resorbing) cells [30,31]. In the presence of ALP (as released by active osteoblasts), however, pyrophosphate ions are hydrolysed (Equation (1)), which can result in localised saturation with respect to phosphate ions resulting in mineral deposition [18,19]. Such an effect seems to have been in operation here, since despite the fact that a steady reduction in the quantity of amorphous calcium pyrophosphate was noted in the cement with time (Fig. 4), considerably more new bone was formed in the defect filled with the pyrophosphate cement than in the orthophosphate cement or the trabecular control (Fig. 2a).

Other groups have investigated the use of higher chain calcium polyphosphate ceramics as bone graft replacements [32,33], however, the potential drawback with these materials is that as degradation occurs the tripolyphosphate ions ($P_3O_{10}^{5-}$) are broken down to form one orthophosphate anion and one pyrophosphate anion (Equation (2)). The continuous production of pyrophosphate

anions during the dissolution of calcium polyphosphate could ultimately interfere with the deposition of new bone and may explain why these materials have not found widespread clinical use.

$$P_3O_{10}{}^{5-} + H_2O \rightarrow PO_4{}^{3-} + P_2O_7{}^{4-} + 2H^+ \quad (2)$$

These previously reported calcium polyphosphate materials [32,33] are also of significantly lower solubility than the amorphous materials deposited within the cement matrix. Consequently, one might expect a slower rate of dissolution into the surrounding media. This also may suggest that the ALP interacts with condensed phosphates that are in solution and not when they are bound in a solid form, which is contrary to previous reports, which have shown that ALP is capable of binding to calcium pyrophosphate salts and to some extent can mediate crystal dissolution [34]. To test this hypothesis, *in vitro* experiments were performed where ALP was exposed directly to calcium pyrophosphate and separated from the surface by a semipermeable membrane. These experiments showed no difference between extents of degradation when the ALP did or did not have access to the crystal surface. This suggests that the binding of ALP to the surface of the calcium pyrophosphate material was not a significant factor (Fig. 5b) and that the accelerated dissolution of the amorphous calcium pyrophosphate salt occurs as a result of Le Chatelier's principle, whereby the removal of pyrophosphate ions from solution, and the subsequent removal of orthophosphate by mineralisation, drive forward the dissolution process (Equation (3)).

$$Ca_2P_2O_7 \cdot 4H_2O \rightleftharpoons 2Ca^{2+}(aq) + P_2O_7{}^{4-}(aq) + 4H_2O \overset{ALP}{\rightleftharpoons} 2Ca^{2+}(aq) + 2PO_4{}^{3-}(aq) + 3H_2O + 2H^+ \quad (3)$$

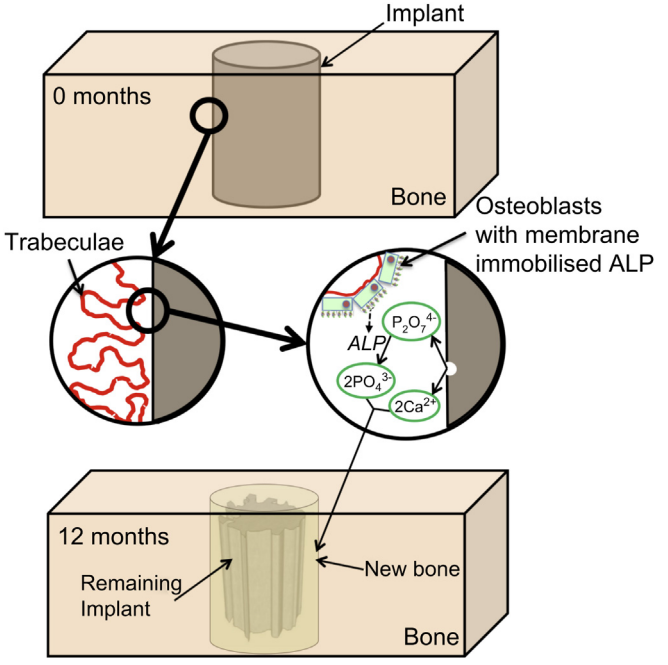

Fig. 6. A schematic diagram illustrating the enzyme mediated degradation and bone formation around the pyrophosphate modified brushite cement. Following implantation, the ceramic material forms an intimate attachment to surrounding bone (upper diagram). Osteoblasts line the surface of the surrounding trabecular bone and their plasma membranes are enriched with respect to ALP (central diagram). As the pyrophosphate ions are dissolved from the cement, they are cleaved by the ALP, which decreases saturation with respect to calcium pyrophosphate and enhances cement dissolution. As the cleavage process also results in supersaturation with respect to HA, new bone formation occurs in the place of the degraded ceramic. After 12 months of implantation there is an extensive cement degradation and new bone formation.

The release of condensed phosphates from amorphous granules and their subsequent hydrolysis by ALP has been proposed to be the mechanism by which bone mineralisation occurs in the body [35] and this method has been used to induce mineralisation within collagen gels [36]. It is possible, therefore, that this material exploits this biological process to enhance bone deposition. We can surmise that the incorporation of amorphous calcium pyrophosphate in bone replacement materials may be an effective way of delivering pyrophosphate species to the locality of the implant/new bone interface. These pyrophosphate species may then be cleaved by the osteoblast associated enzyme (ALP) to provide enzymatically controlled levels of the inorganic components for mineralisation (Fig. 6).

5. Conclusion

Here we have demonstrated that the modification of calcium phosphate biomaterials with amorphous calcium pyrophosphate can effectively enhance the rate of bone formation *in vivo*. The amorphous pyrophosphate phase acted to prevent long-term stability by inhibiting brushite conversion to hydroxyapatite. The pyrophosphate was preferentially dissolved from the hardened ceramic material and an *in vitro* model demonstrated that this was due to accelerated dissolution through cleavage by enzymes with pyrophosphatase activity. The localised removal of the pyrophosphate ions was associated with supersaturation with respect to hydroxyapatite and this was shown to aid bone mineralisation. This is the first example of a cement bone replacement, that contains a phase that may act as a substrate for an enzyme associated with the mineralisation process and can thereby enhance bone accrual. This represents a change in focus in the development of ceramic bone graft replacements, by designing a material to induce a specific biological reaction, rather than implanting a material that mimics a component of a biological structure with little interaction with biological processes at the implant site.

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